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Thermophilic methanol utilization by sulfate reducing bacteria

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# 2

# Methanol degradation in defined mixed cultures of thermophilic anaerobes in the presence of sulfate

Heleen P. Goorissen, Alfons J. M. Stams, and Theo A. Hansen

**In continuous culture experiments the possible conversions performed by a microbial population of a sulfidogenic, thermophilic bioreactor fed with methanol were studied. Mixed cultures of selected organisms were used, representing the different pathways followed by various trophic groups of microorganisms. Assuming Monod-type growth kinetics, the substrate coefficient, $K_s$, and the specific growth rate, μmax, are of crucial importance in the prediction of which bacterial population will become predominant. Moreover, substrate competition will be highly affected by the sensitivity of the organisms to sulfide. Aggregation, which is an important characteristic in bioreactor systems, was not taken into account in our experiments. Our results show that direct competition for methanol between a homoacetogen (*Moorella thermoautotrophica*) and a sulfate reducer is in favor of the sulfate reducer (*Desulfotomaculum kuznetsovii*) due to its affinity for methanol. Methanogenesis as a result of hydrogen transfer between *D. kuznetsovii* and a hydrogen consuming methanogenic archaeon (*Methanothermobacter thermo-autotrophicus*) occurred only below 5 mM total sulfide. A similar result was obtained when *M. thermoacetica* was grown on methanol in the presence *of Mb. thermo-autotrophicus*.**

**Interestingly, *D. kuznetsovii* could coexist with a non-methanol utilizing sulfate reducer (*Thermodesulfovibrio* species). Our data show that it is possible to maintain a dominant sulfate-reducing process with methanol as electron donor at 60 °C in mixed continuous cultures.**

*Submitted for publication*

## Introduction

Methanol is a suitable substrate for the removal of inorganic sulfur compounds from off-gases in anaerobic bioreactors operated at 60 °C or higher (Weijma, 2000). Under thermophilic conditions, methanol can be used by several groups of microorganisms. The presence of sulfate in the reactor leads to sulfide production with concomitant conversion of methanol to $CO_2$ by sulfate-reducing bacteria. Homoacetogens can use methanol in the presence of $CO_2$ for the production of acetate or butyrate. Direct methanogenesis from methanol has not been observed at this temperature. However, methanogens can play a role as hydrogen scavengers in a syntrophic association with methanol utilizers. Theoretically, in a sulfidogenic process methanol might be used directly as electron donor by sulfate-reducing bacteria, or indirectly via the combined activities of microorganisms. The conversion of methanol to sulfate is energetically a more favorable process than the production of methane or acetate from methanol. Thus, in the absence of an external electron acceptor, the formation of methane is more favourable than acetate formation. The conversion of methanol to $H_2/CO_2$ is only energetically favorable at low hydrogen concentrations and therefore depends upon syntrophic hydrogen utilization (for free Gibbs energy yields per reaction, see Table 2.1). Very little is known about thermophilic syntrophic methanol degradation. To our knowledge only two thermophilic sulfidogenic syntrophic associations with methanol have been described and partially characterized. Davidova & Stams (1996) enriched a stable thermophilic sulfate reducing culture from granular sludge of a methanogenic bench-scale reactor. Methanol was degraded syntrophically to acetate, $H_2/CO_2$ and formate by a homoacetogen. The isolated syntrophic sulfate-reducer was unable to use methanol, but could use hydrogen and formate as growth substrates. This strain was not fully characterized, but will be referred to as "*Thermodesulfovibrio* species" in this paper. Another syntrophic association with methanol was obtained from a sulfidogenic EGSB reactor operated with methanol at 65 °C (Weijma, 2000). In this reactor, methanol degrading *Desulfotomaculum* species were present, but part of the methanol was uitlized by other species. Recently, a methanol degrader from this reactor was characterized as a *Thermotoga* species, *Thermotoga lettingae* (Balk, *et al*., 2002) This strain degrades methanol slowly, but degradation rates increased by the addition of thiosulfate or a methanogen. *T. lettingae* was also able to grow on methanol in coculture with, *Thermodesulfovibrio yellowstonii*.

Here, we report for the first time on the kinetics of syntrophic interactions in defined cocultures of a methanol utilizing thermophilic sulfate-reducer *Desulfotomaculum kuznetsovii* and a hydrogenotrophic methanogenic archaeon or another sulfate reducer.

**Table 2.1.** Reactions and related reactions possibly involved in the anaerobic degradation of methanol. Gibbs free energy changes are calculated from Thauer *et al.* (1977)

| Reaction | ΔG º' (kJ/reaction) |
|---|---|
| $4\ CH_3OH + 3\ SO_4^{2-} \rightarrow 4\ HCO_3^- + 3\ HS^- + H^+$ | -364.4 |
| $CH_3OH + HSO_3^- \rightarrow HCO_3^- + HS^- + H_2O + H^+$ | -108.8 |
| $4\ CH_3OH + 2\ HCO_3^- \rightarrow 3\ CH_3COO^- + H^+ + 4\ H_2O$ | -221.6 |
| $CH_3COO^- + SO_4^{2-} \rightarrow 2\ HCO_3^- + HS^-$ | -47.6 |
| $CH_3OH + 2\ H_2O \rightarrow HCO_3^- + 3\ H_2 + HS^-$ | +23.0 |
| $4\ H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + H_2O$ | -151.9 |
| $4\ CH_3OH \rightarrow 3\ CH_4 + HCO_3^- + H_2O + H^+$ | -314.6 |
| $CH_3COO^- + H_2O \rightarrow HCO_3^- + CH_4$ | -31.0 |
| $4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3\ H_2O$ | -135.6 |

## Materials and methods

**Strains.** *Desulfotomaculum kuznetsovii* (DSM 6115) and *Moorella thermoautotrophica* (DSM 1974) were obtained from the Deutsche Sammlung für Mikroorganismen (DSMZ, Braunschweig, Germany). *Methanothermobacter thermoautotrophicus* (formerly *Methanobacterium thermoautotrophicum* ΔH) (DSM 1053) was kindly provided by J.T. Keltjens (University of Nijmegen, The Netherlands). The *Thermodesulfovibrio* species originated from an enrichment culture from Davidova & Stams (1996).

**Media and cultivation.** A bicarbonate buffered basal medium was used containing (g/L): NaCl (7), $NaHCO_3$ (4), $Na_2SO_4$ (2.8), $MgCl_2{\cdot}6H_2O$ (1.2), KCl (0.5), $NH_4Cl$ (0.3), $KH_2PO_4$ (0.2), $CaCl_2$ (0.15), $Na_2S{\cdot}7\text{-}9H_2O$ (0.3). Additions were made from anoxic stock solutions and per liter of medium were added: 0.5 ml vitamin solution according to Stams *et al.* (1983) 1 g yeast-extract, 1 ml trace-element solution SL 6 according to Pfennig & Lippert (1966), and 2 mM acetate as carbon source (if required). Filter sterilized methanol (20 mM, final concentration, or 30 mM in sulfate limiting experiments) was added from anoxic stock solution. Prior to inoculation the gasphase in the headspace was replaced by $N_2/CO_2$ (80%/20%). Bottles were closed with butylrubber stoppers and aluminum caps. In all experiments anaerobic cultivation techniques were used.

**Continuous culture experiment.** Continuous culture experiments were carried out in a 2.5 L Bioflow III fermentation vessel (New Brunswick Sc., Edison NJ) with a 1 L working volume. The reactor, pH probes, and all associated tubing and drip tubes were autoclaved for 30 min at 121 °C prior to use. All tubing connections were made with glass connectors (male-female), and all parts that needed to be connected after autoclaving were wrapped in aluminum foil to maintain sterility. Tubings in the vessel, the headplate and the vessel bottom were made of high-grade stainless steel (RMS). All tubings outside the vessel were made of butylrubber or marprene. During the fermentation the working volume was maintained with a tube at a fixed height, providing overflow of the culture medium in a sterilized 20 L effluent vessel, thereby maintaining constant level. The culture was stirred at 100 rpm during operation and a New Brunswick Sc. (Edison, NJ) motor and speed controller were used to maintain the speed of the stainless steel impeller. Temperature was maintained at 60 °C by constantly circulating water through the round-bottomed-water-jacketed vessel. The pH was maintained at 7.5 by addition of 0.1 M sodium hydroxide to the fermentation by a single speed peristaltic pump (Watson-Marlow Bredel, Falmouth, UK). Autoclavable pH probes (Ingold, Mettler Toledo AG, Urdorf, Switzerland) suitable for applications with high sulfide concentrations were connected through the headplate. Culture medium was pumped through a drip tube connected to the headplate to prevent backgrowth of the culture medium. To control the substrate flow rates, Watson-Marlow pumps (Watson Marlow Bredel, Falmouth, UK) were used. The feed carboy was maintained under slight overpressure with $N_2/CO_2$ that had passed through a sterilized filter. Samples for analyses were taken with nitrogen flushed syringes through a bytulrubber septum in the headspace. Cultures were supposed to be in a steady state after five volume changes. After every experiment with *D. kuznetsovii* all butylrubber tubings, filters and Teflon parts were replaced by new ones.

**Analytical procedures.** Culture optical density was determined in a Starcoll colorimeter (R&D Mechatronics) at a wavelength of 660 nm. All optical density measurements were made immediately after sampling and samples were diluted in medium if necessary. Cells in culture samples were counted using a Bürker-Türk counting chamber. Methanol, methane, and fatty acids were analyzed by GC as described previously (Heijthuysen & Hansen, 1989). Sulfide was determined colorimetrically using the methylene blue method (Trüper & Schlegel, 1064).

## Results

### Direct competition for methanol

***Desulfotomaculum kuznetsovii* and *Moorella thermoautotrophica*.**

*D. kuznetsovii* cells obtained from batch cultures grown on methanol/sulfate/yeast extract, washed in medium and pre-incubated at 60 ºC did not convert methanol, despite the use of strict anaerobic techniques throughout the manipulations. Therefore, methanol conversion kinetics of *D. kuznetsovii* were determined in a methanol limited continuous culture. At steady state ($D = 0.022\ h^{-1}$) the methanol concentration was below the detection limit (below 100 µM). The medium flow was stopped when steady state was reached and methanol (20 mM) was added. Product formation and substrate consumption were followed in time (Fig. 2.1). Methanol consumption occurred at a rate of approximately 170 nmol $min^{-1}$ mg $protein^{-1}$. The apparent $K_m$ value for *D. kuznetsovii* was approximately 1 mM as calculated from the substrate depletion curve.

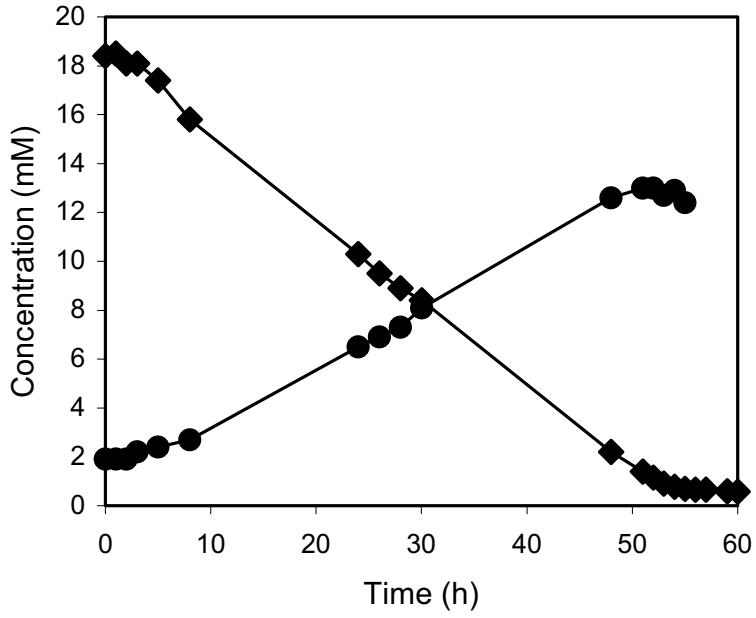


**Figure 2.1.** Methanol consumption in a steady state culture of *D. kuznetsovii.* Symbols: ◆, methanol; ●, sulfide

Due to contamination of our fermentors with extremely heat resistant *D. kuznetsovii* spores, we were not able to grow *M. thermoautotrophica* as a pure culture in the chemostat. When *D. kuznetsovii* and *M. thermoautotrophica* were grown together in a methanol limited continuous culture, the competition for the substrate resulted in a dominancy of the sulfate-reducing process (Fig. 2.2). A fermentor was inoculated with 4% of fresh cultures from both organisms, pregrown on methanol. After a batch phase of 88 hours, medium supply was turned on. A few mmoles of acetate were produced, but at steady state no acetate was detectable anymore. Steady state cultures at a dilution rate of $0.02\ h^{-1}$ with 20 mM methanol in the medium reservoir, contained 0.6 mM methanol. Microscopic examination of the steady state culture revealed that *D. kuznetsovii*

was the dominant microorganism in the fermentor. In batch cultures, growth on acetate by *D. kuznetsovii* is poor, yielding cultures with lower optical densities compared to the mixed culture (results not shown). These observations strongly suggest that sulfate reduction occurred directly from methanol and not indirectly via acetate. Sulfide toxicity experiments in batch culture revealed that sulfide did not inhibit growth of both organisms at concentrations below 10 mM. Because the sulfide concentration in the fermentor was kept below 10 mM, and because the culture was methanol limited, the affinity for methanol was considered to be the crucial parameter in the competition process.

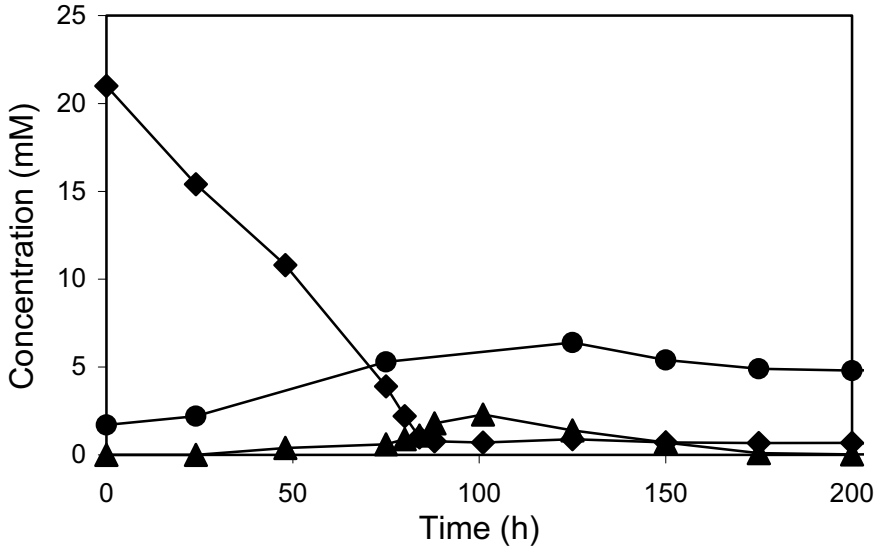


**Figure 2.2.** Competition for methanol between *D. kuznetsovii* and *M. thermoautrophica*. Symbols: ◆, methanol; ●, sulfide; ▲, acetate

## Syntrophic growth

***Desulfotomaculum kuznetsovii* and *Methanothermobacter thermoautotrophicus***.

Syntrophic growth on methanol was observed when *D. kuznetsovii* was cultivated together with *Mb. thermoautotrophicus* in a sulfate limited culture with sulfide concentrations below 5 mM, as well as in a methanol limited culture. A sulfate limited culture of *D. kuznetsovii* was inoculated at t = 7 with 10% of a culture of *Mb. thermoautotrophicus* pregrown on $H_2/CO_2$ (Fig. 2.3a). When the sulfide concentration was reduced due to sparging with $N_2$ (from t = 6), methane was produced as soon as the sulfide concentration was lowered to 5 mM. In a methanol limited culture (Fig. 2.3b) the methanogen still drives the sulfate reducer to hydrogen transfer, even if methanol concentrations were below 0.5 mM. *Mb. thermoautotrophicus* is unable to use methanol and can produce methane only with $H_2/CO_2$. However, when high sulfide concentrations (above 10 mM) were applied, no methane was produced anymore. The toxicity of sulfide seems to be an important parameter to suppress the methane production. To examine whether methane production could be suppressed irreversibly, sulfide pulses were applied to a sulfate-limited steady state coculture of *D. kuznetsovii* and *Mb. thermoautotrophicus* (fig. 2.3c). It was observed that after an extra supply of sulfide to a concentration of 10 mM, methane production did not return. Whereas after a sulfide pulse resulting in a sulfide concentration of 7 mM, methane production returned to the steady state level within 5 hours.

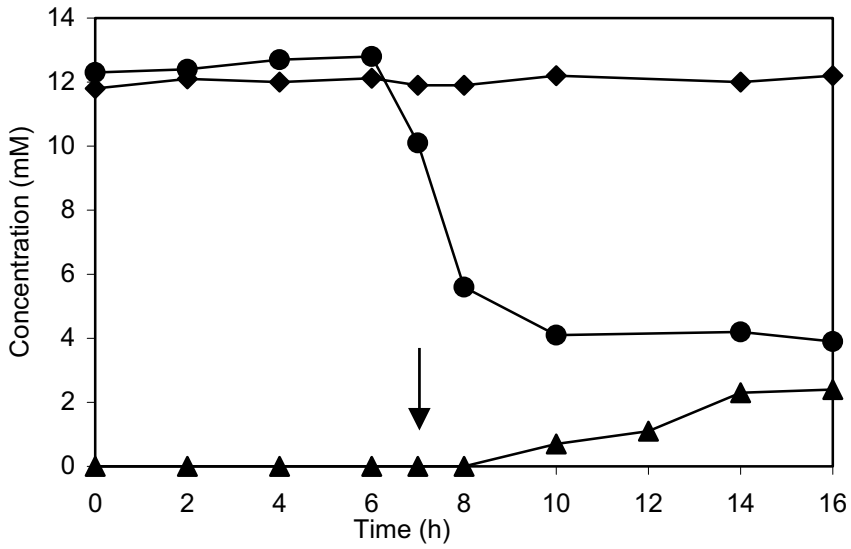


**Figure 2.3a.** Sulfate limited culture of *D. kuznetsovii* and *Mb. thermoautotrophicus* at low sulfide concentration. Arrow indicates inoculation with *Mb. thermoautotrophicus*.
Symbols: ◆, methanol; ●, sulfide; ▲, methane

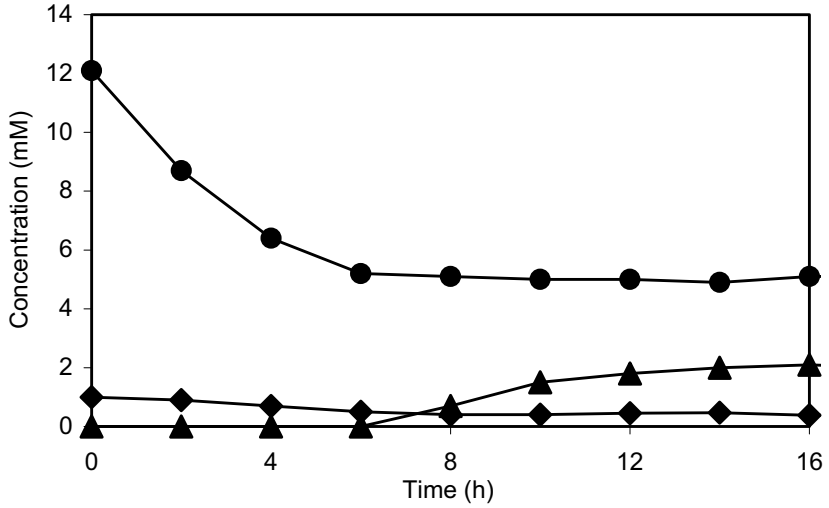


**Figure 2.3b.** Methanol limited culture of *D. kuznetsovii* and *Mb. thermoautotrophicus* at low sulfide concentration. Symbols: ◆, methanol; ●, sulfide; ▲, methane

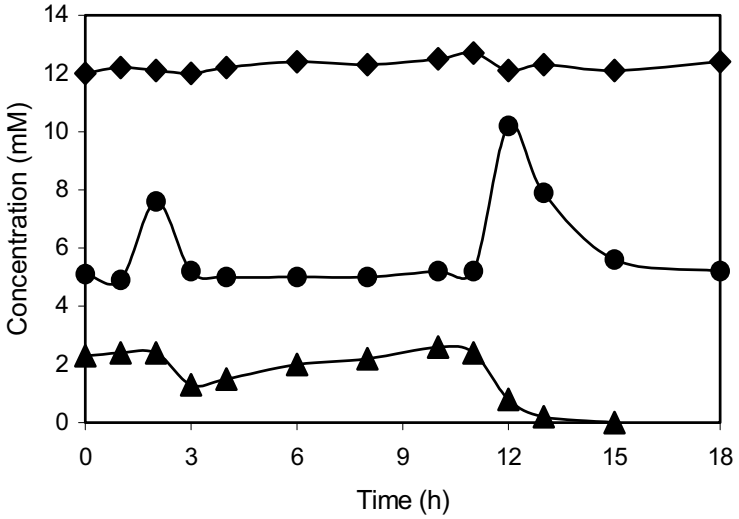


**Figure 2.3c.** Steady state culture of *D. kuznetsovii* and *Mb. thermoautotrophicus* under sulfate limiting conditions. At t = 1 en t= 11 extra sulfide was added.
Symbols: ◆, methanol; ●, sulfide; ▲, methane

***Desulfotomaculum kuznetsovii* and the *Thermodesulfovibrio* species**.

Under methanol limitation a stable, mixed culture of two sulfate reducers was achieved with sulfide concentrations up to 12 mM. Although *D. kuznetsovii* was the dominant organism in this culture during a steady state, *Thermodesulfovibrio*-like cells remained at a constant low level (results not shown).

***Moorella thermoautotrophica* and the*Thermodesulfovibrio* species**.

Because we were not able to grow *M. thermoautotrophica* without *D. kuznetsovii* in a chemostat, we performed batch culture experiments to gain more insight into whether methanol could be used for sulfidogenesis syntrophically. To an exponentially growing acetogenic culture (20 mM methanol) of *M. thermoautotrophica*, 20 mM sulfate was added. The pH of this culture was adjusted to 7.0 with NaOH, followed by inoculation of the culture with 4%(v/v) of the *Thermodesulfovibrio* species. After incubation, 7 mM sulfide was produced. In a control experiment with *M. thermoautotrophica* growing on methanol and sulfate, sulfide was not produced and acetogenesis continued till the substrate was converted completely. The sulfide production in the coculture must be attributed to activity of the *Thermodesulfovibrio* species growing syntrophically with *M. thermoautotrophica*.

**Competition for hydrogen**

***Desulfotomaculum kuznetsovii, Methanothermobacter thermoautotrophicus*, and the *Thermodesulfovibrio* species.**

A coculture of *D. kuznetsovii* and *Mb. thermoautotrophicus* was grown under sulfate limitation and with a constant sulfide concentration of 5 mM. *Mb. thermoautotrophicus* produced 3 mM methane due to syntrophic growth. After a steady state was achieved, the coculture was inoculated with the *Thermodesulfovibrio* species at t = 2. Methane production ceased and *Thermodesulfovibrio*-like cells were detectable in the culture at constant low level after 12 hours of growth (Fig. 2.4). Both at low and at high sulfide concentrations (12 mM), a stable sulfidogenic culture of two sulfate reducers was obtained. These observations were confirmed in batch culture

experiments. A methanogenic culture of *Mb. thermoautotrophicus* growing on $H_2/CO_2$ inoculated with a *Thermodesulfovibrio* species culture pregrown on $H_2/CO_2$, produced 8 mM sulfide instead and no more methane.

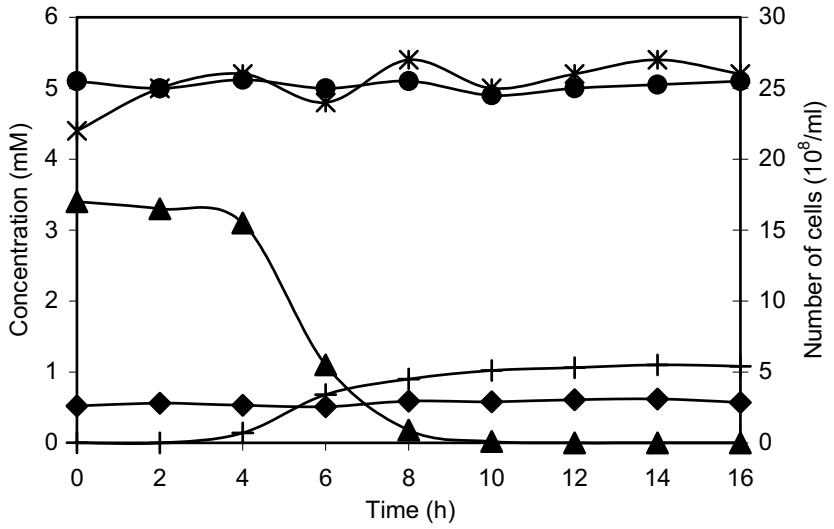


**Figure 2.4.** Sulfate limited culture of *D. kuznetsovii*, *Mb. thermoautotrophicus*, and the *Thermodesulfovibrio* species at low sulfide concentration.
Symbols: ◆, methanol; ●, sulfide; ▲, methane; ✶, *D. kuznetsovii*; +, *Thermodesulfovibrio* species

## Discussion

The direct competition for methanol during methanol limitation between *D. kuznetsovii* and *M. thermoautotrophica* resulted in a dominance of the sulfate reducer. If we assume the ratio $\mu max/K_s$ to be a useful parameter for comparing growth properties of microorganisms on a common substrate, this ratio should be higher for *D. kuznetsovii* than for *M. thermoautotrophica*. The μmax on methanol of *M. thermoautotrophica* is almost twice as high as the $\mu_{max}$ of *D. kuznetsovii*. Thus, the half-saturation constant for methanol of *M. thermoautotrophica* may be assumed to be less than 0.5 mM. At high methanol concentrations, the outcome of the competition may be reverse, due to the higher specific growth rate of *M. thermoautotrophica*.

Syntrophic growth of *D. kuznetsovii* and *Mb. thermoautotrophicus* suggests a central role for hydrogen in the transfer of reducing equivalents, as the methanogen uses only hydrogen as energy substrate. Hydrogen transfer to sulfate reducing bacteria was more pronounced than to methanogens, probably due to differences in affinity, half-saturation constants ($K_s$) and sulfide toxicity. This has also been observed in disintegrated granules from moderately thermophilic UASB reactors. The addition of hydrogen utilizing sulfate reducing bacteria (*Desulfotomaculum* species) resulted in a higher the degradation rate of volatile fatty acids in the granules than the addition of comparable numbers of hydrogen utilizing methanogens (*Methanothermobacter thermo-autotrophicus*) (Schmidt & Ahring, 1993). Our mixed culture experiment with *D. kuznetsovii*, *Mb. thermoautotrophicus*, and the *Thermodesulfovibrio* species confirm these findings.

Hydrogen sulfide concentrations as low as 3.3 mM are already inhibitory for *Desulfotomaculum* species (Min & Zinder, 1990). However, for sulfate reducing bacteria it is observed that sulfide inhibition is reversible (Okabe *et al*., 1995): complete inhibition occured at an $H_2S$ concentration as high as 16.1mM (Reis *et al*., 1992). In our experiments with *D. kuznetsovii* concentrations up to 15 mM $H_2S$ had little effect on the specific growth rate (results not shown). A decrease of 20 % of the specific growth rate was observed at an $H_2S$ concentration of 20 mM. On

the contrary, our experiments showed that sulfide toxicity for methanogenic archaea is irreversible. Uncoupling of growth and activity was not observed for *Mb. thermoautotrophicus*. Visser (1995) showed that a 50% decrease of activity was observed for hydrogenotrophic methanogenesis in methanogenic sludge granules at a concentration of 10 mM sulfide. The high sulfide concentrations (> 30mM) applied in anaerobic bioreactors for the biological desulfurization process (Weijma, 200) guarantee a complete inhibition of methanogenesis by *Methanothermobacter* species.

The coexistence of two sulfate reducers in a stable methanol-limited sulfidogenic culture can be explained by differences in substrate affinity. *D. kuznetsovii* and the *Thermodesulfovibrio* species both can grow on $H_2/CO_2$ and/or formate. No yeast extract was present in the growth medium, thus growth of the *Thermodesulfovibrio* species was due to a syntrophic interaction with *D. kuznetsovii*. Competition for $H_2/CO_2$ and/or formate must be in favor of the *Thermodesulfovibrio* species apparently due to a higher affinity of the *Thermodesulfovibrio* species for $H_2/CO_2$ and/or formate compared to *D. kuznetsovii*.

Substrate competition during syntrophic growth of *D. kuznetsovii* with *Mb. thermo-autotrophicus* and the *Thermodesulfovibrio* species is in favor of the hydrogenotrophic sulfate reducer. Experiments were performed at sulfide concentrations not inhibiting the methanogen, which means that affinity for hydrogen and not toxicity of sulfide is the dominant factor in the competition for hydrogen. Threshold values for hydrogen in sediments are assumed to be lower for sulfate reducers than for methanogens (Weijma, 200).

From our experiments it is clear that sulfate reduction with methanol at 60 ºC in defined mixed cultures of different trophic organisms can be easily maintained. Moreover, competition for substrates between different sulfate reducers and syntrophic growth of different sulfate reducers may be more important in anaerobic environments than has been recognized hitherto.

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